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## Phenylarsine Oxide Increases Intracellular Calcium Mobility and Inhibits $\text{Ca}^{2+}$ -Dependent ATPase Activity in Thymocytes

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A rise in intracellular  $\text{Ca}^{2+}$  levels has been implicated as a regulatory signal for the initiation of lymphocyte proliferation. In the present study the mechanism underlying the elevation of  $[\text{Ca}^{2+}]_i$  induced by phenylarsine oxide (PAO) was investigated in thymocytes. This agent inhibits HIV-1 replication and also NF- $\kappa$ B-mediated activation. It has been reported that the PAO-induced  $\text{Ca}^{2+}$  elevation results from an enhanced plasma membrane calcium permeability in T cells. Here, we present biochemical evidence that the PAO-induced  $\text{Ca}^{2+}$  increase was independent of external  $\text{Ca}^{2+}$ . Consistent with these facts, when  $[\text{Ca}^{2+}]_i$  was depleted by prolonged incubation of the cells in  $\text{Ca}^{2+}$ -free medium, PAO addition did not lead to  $[\text{Ca}^{2+}]_i$  increase. These data indicate the involvement of intracellular organelles of thymocytes as the source of  $\text{Ca}^{2+}$ . Moreover, evidence is presented that PAO inhibited  $\text{Ca}^{2+}$ -dependent ATPase activity from thymocytes and sarcoplasmic reticulum from skeletal muscle. This inhibition was dose-dependent, with a  $\text{IC}_{50}$  of about 30  $\mu\text{M}$  for both preparations of enzyme. The ability of PAO to inhibit  $\text{Ca}^{2+}$ -dependent ATPase represents a novel mechanism of action for this drug. Present data suggest that the PAO-dependent  $[\text{Ca}^{2+}]_i$  increase could be mainly the result of inhi-

bition of  $\text{Ca}^{2+}$ -dependent ATPase. In addition, we describe also a  $\text{Ca}^{2+}$ -dependence for PAO effect on tyrosine phosphorylation. © 1999 Academic Press

**Key Words:** phenylarsine oxide; calcium; thymocytes;  $\text{Ca}^{2+}$ -dependent ATPase.

A variety of early biochemical events occurs upon antigen stimulation of T-cells (for review, see 1). The products of such processes comprise a wide array of molecules referred to as “second messengers.” A rise of  $[\text{Ca}^{2+}]_i$  has been implicated as a regulatory event during the initiation of lymphocyte proliferation (2). The exact role that  $\text{Ca}^{2+}$  might play in this cell activation process is unclear, but the possibility that  $\text{Ca}^{2+}$ /calmodulin could activate calcineurin or other  $\text{Ca}^{2+}$ -dependent proteins has been raised (3). In addition, clear evidence suggests that a network of tyrosine kinases and phosphatases also regulates early steps of T-cell activation (4). The requirement for phosphotyrosine phosphatase CD45 during the T-cell activation process agrees with a reciprocal interplay existing between intracellular kinases and phosphatases (5).

A group of chemical compounds (arsenoxides) acting on SH groups has been used as enzyme inhibitors. One of them, phenylarsine oxide (PAO), is a trivalent arsenical compound (6), which specifically inhibits tyrosine phosphatases in lymphocytes, while leaving p56<sup>lck</sup> and p59<sup>lyn</sup> protein tyrosine kinase activities intact (7). Also, PAO has been recently proposed to be a potential therapeutic drug, since it can inhibit HIV-1 replication in monocytic

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<sup>2</sup> Abbreviations used: PAO, phenylarsine oxide; NF- $\kappa$ B, nuclear factor  $\kappa$ B; EGTA, ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid; dbcAMP, dibutyryl 3'-5'-cyclic AMP;  $\text{IP}_3$ , inositol triphosphate.



cell lines and also NF- $\kappa$ B-mediated activation (8). Evidence has been shown that PAO treatment of T lymphocytes (9) and Jurkat cells (10) inhibits the  $\text{Ca}^{2+}$  increase promoted by an antibody against the surface antigen CD3. However, the treatment of T cells with PAO alone has been observed to cause a dose-dependent increase in  $[\text{Ca}^{2+}]_i$  that was not accompanied by a detectable increase in inositol 1,4,5-trisphosphate production (9,10). This dual effect does not seem related to its ability to inhibit CD45 phosphatase, since in CD45-mutant cells PAO also mobilizes  $\text{Ca}^{2+}$  (9). The present work was undertaken to analyze the intrinsic ability of PAO to increase  $[\text{Ca}^{2+}]_i$  in lymphocytes. We present evidence that PAO exposure produced a rapid and sustained increase in  $[\text{Ca}^{2+}]_i$  in rat thymocytes, which was independent of the presence of external  $\text{Ca}^{2+}$  in the medium. We also show that PAO inhibited  $\text{Ca}^{2+}$ -dependent ATPase activity from endoplasmic reticulum of thymocytes and muscle in a dose-dependent manner. These results suggest that PAO increases  $[\text{Ca}^{2+}]_i$  in lymphocytes through an acute arrest of the endoplasmic reticulum  $\text{Ca}^{2+}$  pump activity, similar to the effect previously described for thapsigargin (11,12). We thus provide the first evidence that PAO-induced  $\text{Ca}^{2+}$  mobilization may be caused by the inhibition of microsomal  $\text{Ca}^{2+}$ -dependent ATPase.

## MATERIALS AND METHODS

### Materials

PAO and Fura-2 acetoxymethyl ester (Fura-2 AM) were obtained from Sigma. PAO was dissolved in DMSO, and the concentration of the solvent in the cell culture never exceeded 0.05% (v/v).

### Preparation of Thymocytes and Subcellular Organelles

Male mice weighing 25–35 g were used. Thymocytes were isolated as described earlier (13). In some experiments, in order to deplete intracellular  $\text{Ca}^{2+}$  stores, a fraction of thymocytes was treated with 5 mM EGTA in Krebs Ringer bicarbonate (KRB) buffer at 37°C for 30 min. After being washed, untreated or treated cells were incubated ( $10 \times 10^6$  cells/ml) in KRB with the additions indicated in the figure legends. The cell viability was assessed by determining the percentage of cells that excluded Trypan blue. Microsomes from thymus and sarcoplasmic reticulum from skeletal muscle were pre-

pared basically as described (14,15). Briefly, thymuses and skeletal muscles from rat were dissected, washed in 0.9% (w/v) NaCl, trimmed, and homogenized (1:5, w/v) in a cold buffer containing 20 mM Tris-HCl, 1 mM EDTA, and 250 mM saccharose, pH 7.4. The homogenate was centrifuged at 1600g for 10 min, and the supernatant obtained was centrifuged again at 12,000g for 30 min. The pellet was discarded and the supernatant was centrifuged again at 100,000g for 1 h and the resulting pellet (microsomes/sarcoplasmic reticulum) was resuspended in the stock buffer using a Teflon-glass homogenizer. The purity of microsomes/sarcoplasmic reticulum preparations was analyzed by specific enzymatic markers, as indicated previously (14,15). Protein concentration was estimated by a modified Lowry method (16).

### Measurement of $[\text{Ca}^{2+}]_i$

Cell loading with Fura-2 AM and measurement of  $[\text{Ca}^{2+}]_i$  were performed basically as in (17), with the modifications previously described (14). Briefly, thymocytes ( $6 \times 10^7$ /ml) were incubated with 2  $\mu\text{M}$  Fura-2 AM in a medium composed of 145 mM NaCl, 5 mM KCl, 1 mM  $\text{MgSO}_4$ , 1 mM  $\text{CaCl}_2$ , 2.5 mM probenecid, 10 mM glucose, 1 mg/ml bovine serum albumin (BSA), and 25 mM Hepes, pH 7.4. After 30–45 min at 37°C, the cells were washed and resuspended in the same medium but lacking Fura-2 AM. Calibration was made by lysing the cells with 50  $\mu\text{M}$  digitonin followed by quenching the fluorescence with 3 mM  $\text{Mn}^{2+}$ , and Fura-2 leakage was determined by the decrease in fluorescence taking place after the addition of 100  $\mu\text{M}$   $\text{Mn}^{2+}$ . Experiments were performed using 0.6 ml of the cell suspension, placed in a thermostatically controlled holder, and fluorescence was measured using a Perkin-Elmer spectrofluorimeter (LS-5). Fura-2 fluorescence ( $\lambda_{\text{exc}}$  at 340 nm and  $\lambda_{\text{emi}}$  at 500 nm) was continuously recorded.

### $\text{Ca}^{2+}$ -Dependent ATPase Activity

ATPase activity from microsomes/sarcoplasmic reticulum was determined by the measurement of release of  $\text{P}_i$  (18). The reaction mixture contained 50 mM KCl, 20 mM Mops (pH 6.8), 10 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$   $\text{CaCl}_2$ , 20  $\mu\text{M}$  ionomycin, and 1–1.5 mg/ml of protein. The mixture was preincubated for 5 min at 37°C with different concentrations of PAO ranging from 5 to 100  $\mu\text{M}$ . The reaction was initiated by the addition of 0.5 mM ATP, incubated for 3 min, and

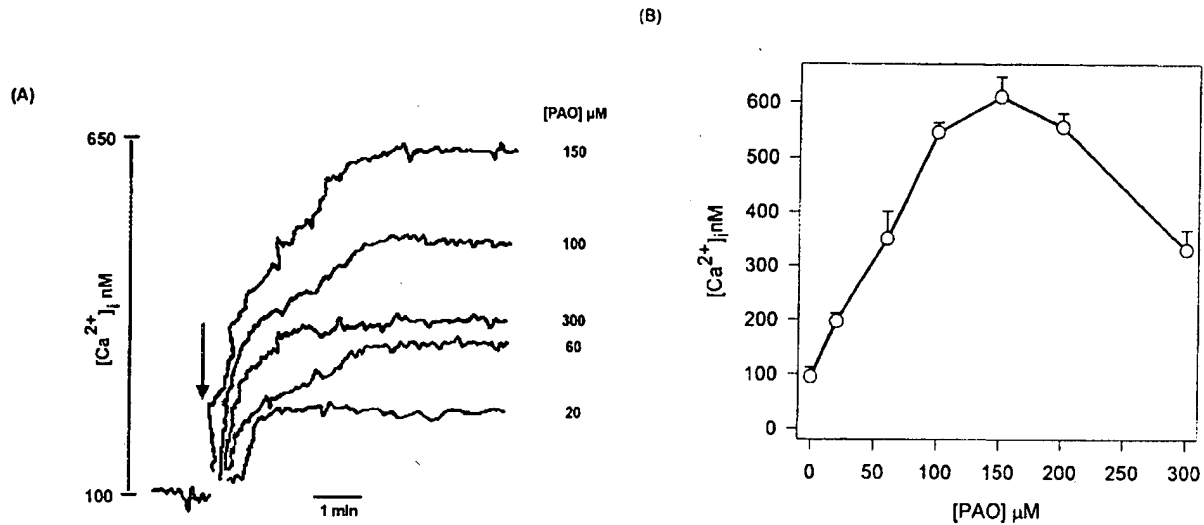


FIG. 1. Time-course (A) and dose-effect (B) of PAO on intracellular  $Ca^{2+}$  levels. (A) After loading with Fura-2, thymocytes were suspended in medium containing 1 mM  $Ca^{2+}$  and the change in fluorescence was recorded during 10 min. PAO was added at the indicated time (arrow) at the concentration shown at the right of each trace. A representative experiment from three independent measurements is shown. The range of variation between groups of experiments was less than 10%. (B) The values of  $[Ca^{2+}]_i$  measured 6 min after PAO addition are plotted as a function of the PAO concentration. Values are means  $\pm$  SE of three independent experiments.

stopped with molybdate/ascorbic acid reactive solution. The absorbance at 630 nm was read after 30 min. Basal,  $Ca^{2+}$ -independent ATPase activity was similarly measured, but in the presence of 0.5 mM EGTA and without added calcium. The subtraction of this last value from that obtained in the presence of calcium corresponds to the  $Ca^{2+}$ -dependent ATPase activity.

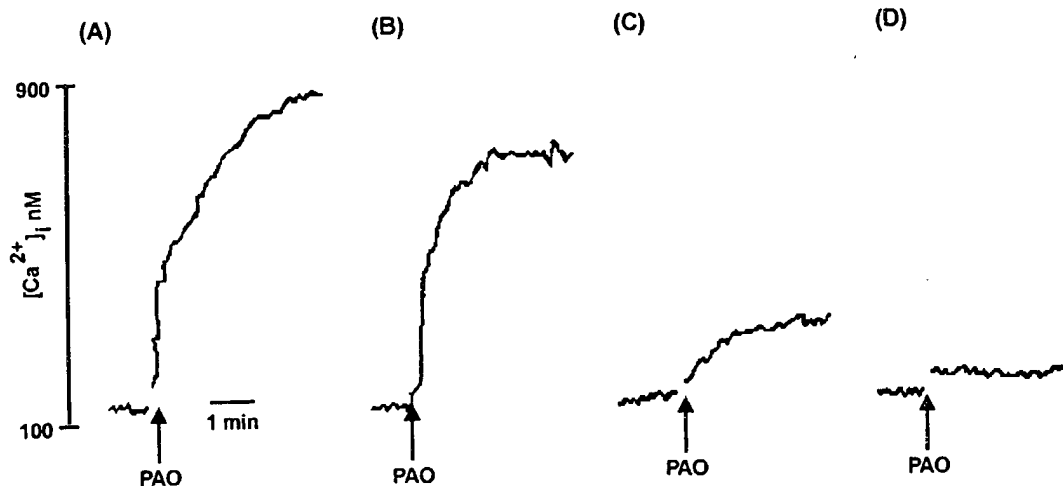
#### *Electrophoresis, Immunoblotting, and Detection of Protein Tyrosine Phosphorylation*

In the experiment described in Fig. 5, the reaction was terminated after 15 min by centrifugation and aspiration of the reaction medium. A volume (90  $\mu l$ ) of lysis buffer containing 50 mM HCl/Tris, pH 7.6, 1% Triton X-100, 300 mM NaCl, 5 mM EDTA, 1 mM  $Na_3VO_4$ , 1 mM PMSF, 10  $\mu g/ml$  of aprotinin and leupeptin, 50  $\mu M$  PAO, and 20 mM NaF was added to the pellet. The cell lysates were resolved by SDS/10%-PAGE by standard procedure as detailed recently (19). Membrane was immunoblotted with anti-phosphotyrosine monoclonal antibody (4G10) (1:2000 final dilution). Bound antibodies were detected by luminol enhanced chemiluminescence, using 4-iodophenol as the enhancer of the chemiluminescence reaction as described by us (19).

## RESULTS

### *Levels of Intracellular Calcium*

Treatment of thymocytes with different doses of PAO, in an external medium containing 1 mM  $Ca^{2+}$ , produced an elevation of  $[Ca^{2+}]_i$  from basal values (about 90–110 nM) to 660 nM. This increase needed 2–4 min to reach a plateau and remained stable for at least 8 min (Fig. 1A). The effect of PAO on  $[Ca^{2+}]_i$  was dose-dependent and reached its maximum level at 150  $\mu M$ . Higher concentrations of PAO did not produce a further increase of  $[Ca^{2+}]_i$  or even decreased it. The half-maximally effective dose for PAO was about 50  $\mu M$  (Fig. 1B). This pattern, and also the required PAO concentrations, was similar to those previously described for lymphocytes and Jurkat cells (9,10). Similar conditions of incubation showed that PAO at 100  $\mu M$  had no effects on thymocyte viability (measured on the basis of lactic dehydrogenase release) after 1 h of treatment (data not shown). When PAO was added to the cells in nominally  $Ca^{2+}$ -free incubation medium (i.e., without added  $Ca^{2+}$  and with 5 mM EGTA), a rapid transient increase in  $[Ca^{2+}]_i$  was observed (Fig. 2A). When the preincubation of thymocytes in  $Ca^{2+}$ -free medium was continued for 5, 10, or 15 min (before PAO addition), a progressive reduction in the PAO-dependent  $[Ca^{2+}]_i$  increase was recorded (Figs. 2B–



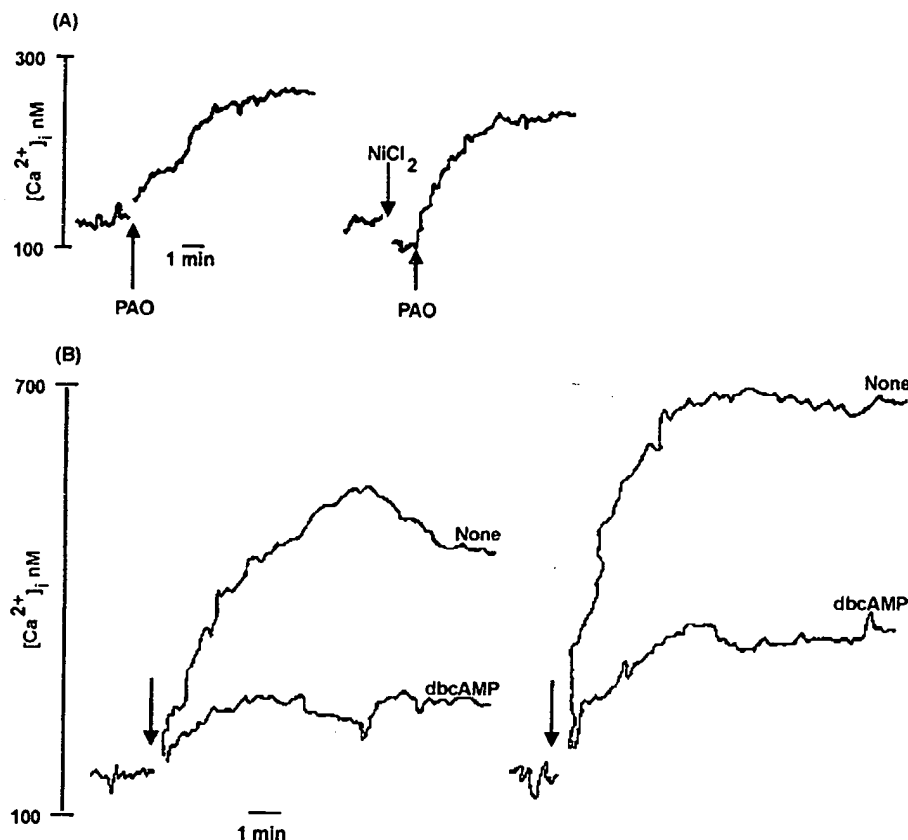
**FIG. 2.** Effect of preincubation with EGTA on PAO-stimulated  $[Ca^{2+}]_i$  levels. Thymocytes were loaded with Fura-2. Before PAO addition, the cells were pretreated in a medium where the extracellular  $Ca^{2+}$  was chelated by the addition of 5 mM EGTA for 0 min (A), 5 min (B), 10 min (C), or 15 min (D). At these times, 150  $\mu$ M PAO was added to the cell suspension, and changes in fluorescence were recorded. Similar traces were observed in three separated experiments.

2D). These data indirectly suggest that the PAO-induced  $[Ca^{2+}]_i$  increase was due to the release of  $Ca^{2+}$  from intracellular stores, since when the cells were depleted of  $Ca^{2+}$  (i.e., after 15 min incubation with 5 mM-EGTA), PAO was unable to mobilize this cation. The further addition of external  $Ca^{2+}$  to these EGTA-treated cells rapidly produced an influx of extracellular  $Ca^{2+}$  inside the cells (data not shown). In order to further analyze the source of the PAO-induced  $[Ca^{2+}]_i$  increase, experiments using  $NiCl_2$ , a well-known blocker of  $Ca^{2+}$  transport through the plasma membrane (20), were performed. The presence of 400  $\mu$ M  $Ni^{+2}$  in the incubation medium did not produce any detectable effect on the PAO-induced  $[Ca^{2+}]_i$  increase (Fig. 3A). This result further confirmed the previous assumption that observable PAO-induced  $[Ca^{2+}]_i$  increase arose from intracellular stores. In a search for other inhibitors of tyrosine phosphatases which could produce a similar action on  $[Ca^{2+}]_i$  levels, we observed that vanadate was without effect (data not shown). It has been previously described that dibutyryl-cyclic AMP (dbcAMP), as well as agents which increase intracellular cAMP (e.g., forskolin and prostaglandin  $E_2$ ), suppresses in a dose-dependent fashion the anti-CD3-induced  $Ca^{2+}$  elevation in T lymphocytes (21). These data prompted us to study whether dbcAMP was able to inhibit the PAO-induced  $[Ca^{2+}]_i$  increase in thymus cells. Figure 3B illustrates that the presence of 1.5 mM dbcAMP abolished almost completely (80–90% inhibition) the  $[Ca^{2+}]_i$  increase

stimulated by different doses of PAO. It is noteworthy that at the analyzed dose of PAO a small fraction of PAO-induced  $[Ca^{2+}]_i$  increase remained not inhibited by dbcAMP. The inhibitory effect of dbcAMP was not canceled by PAO concentrations higher than 150  $\mu$ M and remained constant during the time measurement (8–10 min). A similar inhibitory effect was observed when the cells were previously preincubated for 1–3 h with dbcAMP (data not shown). The mechanism by which cyclic AMP inhibits the agonist-dependent  $Ca^{2+}$  mobilization remains to be clarified, and it could be related to a yet undetermined protein kinase.

#### *Inhibitory Effect of PAO on $Ca^{2+}$ -Dependent-ATPase Activity*

In order to establish the mechanism underlying the PAO-induced  $[Ca^{2+}]_i$  increase, and since the pattern of this phenomenon was found to be very comparable to that described for thapsigargin, we tested the possibility that the  $[Ca^{2+}]_i$  increase could be due to some alteration of  $Ca^{2+}$ -dependent ATPase activity. Previously it had been demonstrated that thapsigargin pretreatment of rat liver microsomes blocked subsequent loading with  $^{45}Ca^{2+}$  and also produced a rapid inhibition of  $Ca^{2+}$ -dependent ATPase activity (11). Also thapsigargin elicited a rapid and dose-dependent increase in  $[Ca^{2+}]_i$  (12). Figure 4 illustrates the effect of PAO on different preparations of  $Ca^{2+}$ -dependent ATPases. The direct measurement of enzymatic ATP hydrolysis revealed that



**FIG. 3.** Effects of  $NiCl_2$  (A) and dibutyl-cyclic AMP (B) on PAO-stimulated  $[Ca^{2+}]_i$  levels. (A) Fura-2-loaded thymocytes were pretreated for 1 min either in the absence (left) or in the presence (right) of  $400 \mu M Ni^{+2}$ , an inhibitor of calcium transport through the plasma membrane. Thereafter, lymphocytes were treated with  $60 \mu M$  PAO (arrow), and the change of fluorescence was recorded. (B) Lymphocytes were preincubated in the absence (none) or in the presence (dbcAMP) of  $2 mM$  dibutyl-cyclic AMP for 10 min. Thereafter, PAO (arrow) at  $100 mM$  (left) or at  $150 \mu M$  (right) was added to cell suspension and the fluorescence was recorded.

PAO inhibited both microsomes/sarcoplasmic reticulum  $Ca^{2+}$ -dependent ATPase with an  $EC_{50}$  value of about  $30 \mu M$  (Fig. 4). Nevertheless, basal  $Ca^{+2}$ -independent ATPase was unaffected after PAO treatment (data not shown).

#### *Intracellular $Ca^{2+}$ and PAO-Dependent Tyrosine Phosphorylation*

Since PAO increases both intracellular  $Ca^{2+}$  levels and tyrosine phosphorylation, acting as a potent inhibitor of tyrosine phosphatases (7,9,10), we have analyzed the potential relationship between  $Ca^{2+}$  levels and PAO-dependent tyrosine phosphorylation. For this purpose, we have depleted  $Ca^{2+}$  from the internal stores by preincubating the cells with EGTA, and after being washed, PAO was added to the cells at the indicated dose for 15 min (Fig. 5). It was observed that  $Ca^{2+}$ -depleted cells present a

lower level of tyrosine phosphorylation in most proteins than untreated cells. These results suggest that intracellular  $Ca^{2+}$  may be required either for the PAO-dependent inhibition of tyrosine phosphatase or for the activation of the corresponding tyrosine kinase. The absence of  $Ca^{2+}$  seems especially relevant for tyrosine phosphorylation of proteins with apparent molecular masses of 129, 112, and 56 kDa (Fig. 5). In fact, although the addition of PAO allows the tyrosine phosphorylation of proteins, it is noteworthy that, at the same PAO concentration used, the presence of  $Ca^{2+}$  induced the tyrosine phosphorylation of specific proteins in a greater extent than its absence. Although the increase of PAO-dependent tyrosine phosphorylation is well known for other cellular systems (7,9,10), the  $Ca^{2+}$  dependence for PAO effect has not been previously reported.

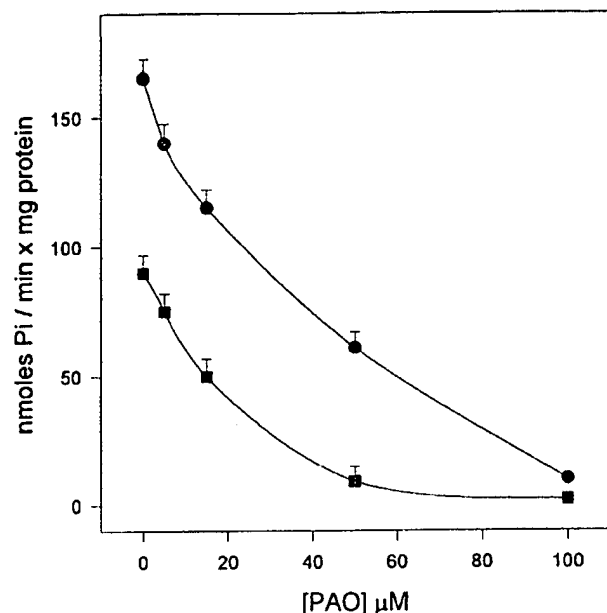


FIG. 4.  $\text{Ca}^{2+}$ -dependent ATPase(s) inhibition by PAO. Microsomes for thymus (■) and muscle sarcoplasmic reticulum (●) were prepared from mice as indicated under Materials and Methods. These subcellular preparations were incubated at  $37^\circ\text{C}$  with the indicated concentrations of PAO for 5 min. The reaction was initiated by the addition of 0.5 mM ATP. After 3 min of incubation, phosphate from the medium was analyzed. Data are the means of three separate experiments performed in triplicate.

## DISCUSSION

The identification of PAO as a specific inhibitor of tyrosine phosphatase makes this compound an important tool for analyzing the intracellular signal transduction process. The wide variety of PAO effects on biological systems includes the inhibition of NADPH-oxidase from macrophages and neutrophils (22,23), tyrosine phosphorylation of phospholipase  $\text{C}\gamma$ -2 (24), TNF-dependent activation of NF- $\kappa$ B (25), and the HIV-1 replication (8).

Present data confirm the previously described PAO-induced  $[\text{Ca}^{2+}]_i$  increase effect in lymphocyte cell lines (10,11,26), extend these observations to a primary cell preparation (such as thymocytes), and offer a mechanism for its interpretation. Mobilization of  $\text{Ca}^{2+}$  frequently exhibits two phases, an initial peak due to its release from intracellular stores and a second phase attributable to an influx of  $\text{Ca}^{2+}$  through the plasma membrane, termed capacitative entry (27). But, at a difference with previous data (10,11,26), our results implicate  $\text{Ca}^{2+}$  released from internal stores as the main component of the ob-

served cytosolic  $\text{Ca}^{2+}$  elevation in thymocytes. This assumption is based on the following observations: first, PAO raised  $[\text{Ca}^{2+}]_i$  in the absence of extracellular calcium (Fig. 2A). Furthermore,  $\text{Ni}^{2+}$ , a blocker of  $\text{Ca}^{2+}$  influx through the plasma membrane, did not alter the PAO-effect on  $[\text{Ca}^{2+}]_i$  (Fig. 3A), discarding the idea that an effect of membrane permeability to this cation could play some role in the PAO-induced  $[\text{Ca}^{2+}]_i$  increase. And third, when stringent experimental conditions (15 min of treatment with 5 mM EGTA) were introduced to deplete intracellular  $\text{Ca}^{2+}$  stores, PAO was unable to elicit any  $[\text{Ca}^{2+}]_i$  increase (Fig. 2D). Previous experiments on lymphocytes (9,10) and on endothelial cells (26), using mild  $\text{Ca}^{2+}$  depletion conditions (i.e., 1.5 mM EGTA treatment during 2 min), showed a partial inhibition of the PAO-dependent  $\text{Ca}^{2+}$  increase, and the authors concluded that PAO also stimulates  $\text{Ca}^{2+}$  influx from the extracellular milieu. However, it may be argued that this effect could be the result of a decreased  $\text{Ca}^{2+}$  reserve in the internal stores, without the participation of  $\text{Ca}^{2+}$  influx through the plasma membrane. In fact, we have found (Figs. 2A–2D) a gradual decrease on  $[\text{Ca}^{2+}]_i$  to occur depending on the period of EGTA treatment, similar to their finding using mild conditions. The potential stimulation by PAO of  $\text{Ca}^{2+}$  influx through the plasma membrane is difficult to prove using external EGTA, since in such nominally  $\text{Ca}^{2+}$ -free medium the depletion of inter-

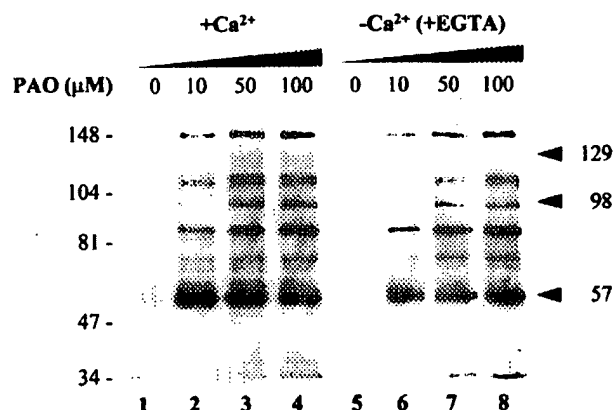


FIG. 5. Effect of PAO on tyrosine phosphorylation by calcium-depleted cells. Control, untreated thymocytes (lanes 1–4), and thymocytes exposed to 5 mM EGTA for 30 min (lanes 5–8) were washed and incubated either without additions (lanes 1 and 5) or with 10  $\mu\text{M}$  (lanes 2 and 6), 50  $\mu\text{M}$  (lanes 3 and 7), and 100  $\mu\text{M}$  (lanes 7 and 8) of PAO for 15 min. After lysis, proteins were separated by SDS-PAGE and phosphorylation on tyrosine was analyzed by immunoblotting with anti-phosphotyrosine mAb (4G10). Two experiments were performed with similar results.

nal  $\text{Ca}^{2+}$  stores provokes a sustained and agonist-independent  $\text{Ca}^{2+}$  influx when external  $\text{Ca}^{2+}$  is added (capacitative entry) (28, and data not shown).

$\text{Ca}^{2+}$  pumps are members of a large superfamily of P-type ATPases and have been classified as endoplasmic reticulum or plasma membrane type  $\text{Ca}^{2+}$ -ATPase (types IIA and IIB, respectively), based on enzymes first identified in animal systems (29,30). We show evidence for the first time that PAO inhibited the  $\text{Ca}^{2+}$ -dependent ATPase from thymus microsomes and sarcoplasmic reticulum (Fig. 4), suggesting that the PAO-induced  $[\text{Ca}^{2+}]_i$  elevation could be the result of a predominant balance toward its extrusion from  $\text{Ca}^{2+}$  stores. This effect is attributable to the inhibition of cytosolic  $\text{Ca}^{2+}$  uptake, which is dependent on  $\text{Ca}^{2+}$ -ATPase from endoplasmic reticulum. In agreement with this possibility, evidence has been presented that PAO does not induce phosphoinositide hydrolysis with the concomitant production of  $\text{InsP}_3$  (9,26,31). Although  $\text{InsP}_3$  is clearly a major regulator of intracellular  $\text{Ca}^{2+}$  handling (32), a variety of experimental evidences suggest that  $\text{InsP}_3$ -independent  $\text{Ca}^{2+}$  regulatory pathways exist as well. For example, resting B-cells stimulated with concanavalin A display a large increase in intracellular  $\text{Ca}^{2+}$  levels, but only a very small release of  $\text{InsP}_3$  (33). Likewise, a dichotomy between  $\text{Ca}^{2+}$  and phosphoinositide hydrolysis has been noted in T-cells (31). The effects described here for PAO are similar to those previously reported for thapsigargin regarding promotion of an  $\text{IP}_3$ -independent  $\text{Ca}^{2+}$  increase (34) and inhibition of  $\text{Ca}^{2+}$ -ATPase (11,12), although the effect elicited by PAO seems less potent and less specific for microsomes than that of thapsigargin. The activity of PAO as an inhibitor of tyrosine phosphatases (7) could be causally related to its ability to inhibit  $\text{Ca}^{2+}$ -dependent ATPase activity reported here. In this context, the plasma membrane  $\text{Ca}^{2+}$  pump is sensitive to the cyclic GMP-dependent protein kinase (35); and the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase activity is also regulated by a kinase system (36). Interestingly,  $\text{Ca}^{2+}$  levels may be directly regulated by protein tyrosine kinases in T-lymphocytes (31) or endothelial cells (26), and it has also been described that phosphorylation down-regulates the store-operated  $\text{Ca}^{2+}$  entry pathway (37).

Therefore, given the potential ability of PAO to interact with the  $\text{Ca}^{2+}$  pump, this compound may constitute a suitable molecular tool for analyzing  $\text{Ca}^{2+}$  homeostasis and offer new insight into the

$\text{Ca}^{2+}$ -induced activation of transcription factors, such as NF- $\kappa$ B and NF-AT (38).

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## REFERENCES

1. Szamel M, Resch K. T-cell antigen receptor-induced signal-transduction pathways activation and function of protein kinases C in T lymphocytes. *Eur J Biochem* 228:1-15, 1995.
2. Gelfand EW, Mills GB, Cheung RK, Lee JW, Grinstein S. Transmembrane ion fluxes during activation of human T lymphocytes: role of  $\text{Ca}^{2+}$ ,  $\text{Na}^+/\text{H}^+$  exchange and phospholipid turnover. *Immunol Rev* 95:59-87, 1987.
3. O'Keefe SJ, Tamura J, Kincaid RL, Tocci MJ, O'Neill EA. FK-506 and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. *Nature* 357:692-694, 1992.
4. Sefton BM, Campbell MA. The role of tyrosine protein phosphorylation in lymphocyte activation. *Annu Rev Cell Biol* 7:257-274, 1991.
5. Cohen P. Signal integration at the level of protein kinases, protein phosphatases and their substrates. *Trends Biochem Sci* 17:408-413, 1992.
6. Kutsumi H, Kawai K, Richard B, Johnston Jr, Kazuhito Rokutan. Evidence for participation of vicinal dithiols in the activation sequence of the respiratory burst of human neutrophils. *Blood* 85:2559-2569, 1995.
7. Garcia-Morales P, Minami Y, Luong E, Klausner RD, Samelson LE. Tyrosine phosphorylation in T cells is regulated by phosphatase activity: studies with phenylarsine oxide. *Proc Natl Acad Sci USA* 87:9255-9259, 1990.
8. Arbault S, Edeas M, Legrand-Poels S, Sojic N, Amatore C, Piette J, Best-Belpomme M, Lindenbaum A, Vuillaume M. Phenylarsine oxide inhibits ex vivo HIV-1 expression. *Biomed Pharmacother* 51:430-438, 1997.
9. Fletcher MC, Samelson LE, June CH. Complex effects of phenylarsine oxide in T cells. Induction of tyrosine phosphorylation and calcium mobilization independent of CD45 expression. *J Biol Chem* 268:23697-23703, 1993.
10. Deckert M, Aussel C, Bernard A, Breittmayer JP. Phenylarsine oxide and phorbol myristate acetate inhibit the CD3-induced rise of cytosolic  $\text{Ca}^{2+}$  in Jurkat cells by refilling internal  $\text{Ca}^{2+}$  stores. *Biochem J* 297:567-72, 1994.
11. Thastrup O, Cullen PJ, Drobak BK, Hanley MR, Dawson AP. Thapsigargin, a tumor promoter, discharges intracellular  $\text{Ca}^{2+}$  stores by specific inhibition of the endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase. *Proc Natl Acad Sci USA* 87:2466-2470, 1990.
12. Kijima Y, Ogunbunmi E, Fleischer S. Drug action of thapsigargin on the  $\text{Ca}^{2+}$  pump protein of sarcoplasmic reticulum. *J Biol Chem* 266:22912-22918, 1991.
13. Martin F, Gualberto A, Sobrino F, Pintado E. Thimerosal



- induces calcium mobilization, fructose 2,6-bisphosphate synthesis and cytoplasmic alkalization in rat thymus lymphocytes. *Biochim Biophys Acta* 1091:110-114, 1991.
14. Baquero-Leonis D, Pintado E. Calcium release induced by inositol 1,4,5-trisphosphate in thymocyte microsomes. Inhibition by barium and strontium. *Cell Calcium* 10:451-455, 1989.
  15. Pintado E, Scarpa A, Cohn M. Calcium transport and ATPase activities of sarcoplasmic reticulum with adenosine 5'-O-(2-thiotriphosphate) diastereomers as substrates. *J Biol Chem* 257:11346-11352, 1982.
  16. Markwell MA, Haas SM, Bieber LL, Tolbert EN. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* 87:206-210, 1978.
  17. Grynkiewicz G, Poenie M, Tsien RY. A new generation of  $\text{Ca}^{2+}$  indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440-3450, 1985.
  18. Leloir LF, Cardini CE. Characterization of phosphorus compounds by acid lability. In *Methods in Enzymol*, Vol 3 (Colowick SP, Kaplan NO, Eds.). New York: Academic Press, pp 840-850, 1957.
  19. Carballo M, Marquez G, Conde M, Martin-Nieto J, Monteseirin J, Conde J, Pintado E, Sobrino F. Characterization of calcineurin in human neutrophils. Inhibitory effect of hydrogen peroxide on its enzyme activity and on NF- $\kappa$ B DNA binding. *J Biol Chem* 274:93-100, 1999.
  20. Merritt JE, Jacob R, Hallam TJ. Use of manganese to discriminate between calcium influx and mobilization from internal stores in stimulated human neutrophils. *J Biol Chem* 264:1522-1527, 1989.
  21. Papadogiannakis N, Nordstrom TE, Andersson LC, Wolff CH. cAMP inhibits the OKT3-induced increase in cytoplasmic free calcium in the Jurkat T cell line: the degree of inhibition correlates inversely with the amount of CD3 binding ligand used. *Eur J Immunol* 19:1953-1956, 1989.
  22. Conde M, Chiara MD, Pintado E, Sobrino F. Modulation of phorbol ester-induced respiratory burst by vanadate, genistein, and phenylarsine oxide in mouse macrophages. *Free Radical Biol Med* 18:343-348, 1995.
  23. Le Cabec V, Maridonneau-Parini Y. Complete and reversible inhibition of NADPH oxidase in human neutrophils by phenylarsine oxide at a step distal to membrane translocation of the enzyme subunits. *J Biol Chem* 270:2067-2073, 1995.
  24. Yanaga F, Asselin J, Schieven GL, Watson SP. Phenylarsine oxide inhibits tyrosine phosphorylation of phospholipase C gamma 2 in human platelets and phospholipase C gamma 1 in NIH-3T3 fibroblasts. *FEBS Lett* 368:377-380, 1995.
  25. Singh S, Aggarwal BB. Protein-tyrosine phosphatase inhibitors block tumor necrosis factor-dependent activation of the nuclear transcription factor NF- $\kappa$ B. *J Biol Chem* 270:10631-10639, 1995.
  26. Fleming I, Fisslthaler B, Busse R. Interdependence of calcium signaling and protein tyrosine phosphorylation in human endothelial cells. *J Biol Chem* 271:11009-11015, 1996.
  27. Putney JW. Capacitative calcium entry revisited. *Cell Calcium* 11:611-624, 1990.
  28. Montero M, Garcia-Sancho J, Alvarez J. Comparative effects of cytochrome P-450 inhibitors on  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  entry induced by agonists or by emptying the  $\text{Ca}^{2+}$  stores of human neutrophils. *Biochim Biophys Acta* 1177:127-133, 1993.
  29. Moller JV, Juul B, le Maire M. Structural organization, ion transport, and energy transduction of P-type ATPases. *Biochim Biophys Acta* 1286:1-5, 1996.
  30. Carafoli E. The  $\text{Ca}^{2+}$  pump of the plasma membrane. *J Biol Chem* 267:2115-2118, 1992.
  31. Niklinska BB, Yamada H, O'Shea JJ, June CH, Ashwell JD. Tyrosine kinase-regulated and inositol phosphate-independent  $\text{Ca}^{2+}$  elevation and mobilisation in T cells. *J Biol Chem* 267:7154-7159, 1992.
  32. Berridge MJ. Inositol trisphosphate and diacylglycerol as second messengers. *Biochem J* 220:345-360, 1984.
  33. Bijsterbosch MK, Klaus GG. Concanavalin A induces  $\text{Ca}^{2+}$  mobilisation, but only minimal inositol phospholipid breakdown in mouse B cells. *J Immunol* 137:1294-1299, 1986.
  34. Jackson TR, Patterson SI, Thastrup O, Hanley MR. A novel tumour promoter, thapsigargin, transiently increases cytoplasmic free  $\text{Ca}^{2+}$  without generation of inositol phosphates in NG115-401L neuronal cells. *Biochem J* 253:81-86, 1988.
  35. Vrolix M, Raeymaekers L, Wuytack F, Hofmann F, Casteels R. Cyclic GMP-dependent protein kinase stimulates the plasmalemmal  $\text{Ca}^{2+}$  pump of smooth muscle via phosphorylation of phosphatidylinositol. *Biochem J* 255:855-863, 1988.
  36. Tada M, Kadoma M. Regulation of the  $\text{Ca}^{2+}$  pump ATPase by cAMP-dependent phosphorylation of phospholamban. *Bioessays* 10:157-163, 1989.
  37. Montero M, Garcia-Sancho J, Alvarez J. Phosphorylation down-regulates the store-operated  $\text{Ca}^{2+}$  entry pathway of human neutrophils. *J Biol Chem* 269:3963-3967, 1994.
  38. Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI. Differential activation of transcription factors induced by  $\text{Ca}^{2+}$  response amplitude and duration. *Nature* 386:855-858, 1997.